

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Degenerative Myelopathy in German Shepherd Dog: comparison of two molecular assays for the identification of the SOD1:c.118G>A mutation.

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/141734> since 2015-09-04T09:02:19Z

Published version:

DOI:10.1007/s11033-013-2904-9

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

Molecular Biology Reports

Degenerative Myelopathy in German Shepherd Dog: comparison of two molecular assays for the identification of the SOD1:c.118G>A mutation --Manuscript Draft--

Manuscript Number:	MOLE-7752
Full Title:	Degenerative Myelopathy in German Shepherd Dog: comparison of two molecular assays for the identification of the SOD1:c.118G>A mutation
Article Type:	Manuscript
Keywords:	German Shepherd Dog; SOD1; Primer-Extension Reaction; Real Time PCR; Allelic discrimination.
Corresponding Author:	Alessandra Dalmasso ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	
Corresponding Author's Secondary Institution:	
First Author:	Maria Teresa Capucchio
First Author Secondary Information:	
Order of Authors:	Maria Teresa Capucchio Veronica Spalenza Elena Biasibetti Maria Teresa Bottero Roberto Rasero Alessandra Dalmasso paola sacchi, PhD
Order of Authors Secondary Information:	
Suggested Reviewers:	<p>Martin Pumarola Department of Animal Medicine and Surgery, Veterinary Clinical Science, Autonomous University of Barcelona, Barcelona, 08193 Bellaterra (Barcelona), Spain. marti.pumarola@uab.es</p> <p>Fabio Pablo de Souza Departamento de Zootecnia, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Via de Acesso Prof. Paulo Donato Castellane, Jaboticabal, SP 14884-900, Brazil fabiopablos@hotmail.com</p> <p>Brian Summers Royal Veterinary College bsummers@rvc.ac.uk</p> <p>Davide Schiffer Universita di Torino davide.schiffer@unito.it</p> <p>Bartolomeo Biolatti Universita di Torino bartolomeo.biolatti@unito.it</p> <p>Carlo Cantile</p>

Degenerative Myelopathy in German Shepherd Dog: comparison of two molecular assays for the identification of the *SOD1:c.118G>A* mutation

Maria Teresa Capucchio¹, Veronica Spalenza¹, Elena Biasibetti, Maria Teresa Bottero, Roberto Rasero, Alessandra Dalmasso, Paola Sacchi

Genotyping for Degenerative Myelopathy in German Shepherd Dog

Department of Veterinary Sciences, University of Turin, Italy

Corresponding author: Alessandra Dalmasso, via Leonardo da Vinci, 44 - 10095 Grugliasco, Italy, alessandra.dalmasso@unito.it.

¹These Authors contributed equally to this work

Abstract

Degenerative myelopathy is a late-onset, slowly progressive degeneration of spinal cord white matter which is reported primarily in large breed dogs. The missense mutation *SOD1:c.118G>A* is associated with this pathology in several dog breeds, including the German Shepherd Dog. The aims of the present study were to develop a tool for the rapid screening of the *SOD1* mutation site in dogs and to evaluate the association of the polymorphism with degenerative myelopathy in the German Shepherd breed. Two different techniques were compared: a minisequencing test and a Real-Time PCR Allelic Discrimination assay. Both approaches resulted effective and efficient. A sample of 47 dogs were examined. Ten subjects presented the symptoms of the illness; for one of them the diagnosis was confirmed by postmortem investigations and it resulted to be an A/A homozygote. In another clinically suspected dog, heterozygote A/G, the histopathological examination of the medulla showed moderate axon and myelin degenerative changes. German Shepherd Dog shows a frequency of the mutant allele equal to 0.17, quite high being a high-risk allele. Because canine degenerative myelopathy has a late onset in adulthood and homozygous mutant dogs are likely as fertile as other genotypes, the natural selection is mild and the mutant allele may reach high frequencies. A diagnostic test, easy to implement, may contribute to control the gene diffusion in populations. The *SOD1:c.118G>A* mutation could be a useful marker for breeding strategies intending to reduce the incidence of degenerative myelopathy.

Keywords: German Shepherd Dog, SOD1, Primer-Extension Reaction, Real Time PCR, Allelic discrimination.

1 Introduction

2 Degenerative myelopathy (DM) is a late-onset, slowly progressive degeneration of the spinal cord
3
4 white matter which is reported primarily in large-breed dogs. The condition has been described in
5
6 German Shepherd Dog (GSD) as well as in other breeds like Siberian Husky, Miniature Pooodle,
7
8
9 Boxer, Pembroke Welsh Corgi, Chesapeake Bay Retriever, and Rhodesian Ridgeback [1]. Dogs
10
11 with DM show an insidiously progressive ataxia and paresis of the pelvic limbs, ultimately leading
12
13 to loss of the ability to ambulate within 6 to 12 months of the onset of signs. Definitive diagnosis of
14
15
16 DM is confirmed by postmortem histological examination of the spinal cord [2]. There is no sex
17
18 bias. Most dogs are at least 8 years old before the onset of clinical signs [1]. DM appears to be an
19
20
21 autosomal, recessive, and incompletely penetrant disease [3].
22
23

24
25 Human amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by
26
27 adult-onset, progressive loss of motor neurons in the motor cortex, brainstem and spinal cord (lower
28
29 motor neurons), leading to muscle atrophy, generalized paralysis, respiratory depression, and
30
31 inevitable death [4, 5, 6, 7]. In 1993, mutations in superoxide dismutase 1 (*SOD1*) gene were
32
33 associated with familial ALS [8]. So far, more than a hundred *SOD1* different mutations have been
34
35 identified in ALS patients. Mutations in other genes have been reported to account for a small
36
37 number of cases of familial ALS [9].
38
39

40
41 Interestingly, relationship between the missense mutation *SOD1:c.118G>A* and canine DM has
42
43 recently been reported [3]. This mutation was associated with DM in several dog breeds, including
44
45 the GSD. The related SNP could be a useful marker for breeding strategies intending to reduce the
46
47 incidence of DM.
48
49

50
51 The first aim of the present study was to develop a tool for rapid screening of the *SOD1* mutation
52
53 site in dogs comparing two different molecular approaches: i) minisequencing (or Primer-Extension
54
55 Reaction - PER) and ii) Real-Time PCR Allelic Discrimination TaqMan MinorGroove Binding
56
57

(MGB). The second aim was to evaluate the association of *SOD1c.118G>A* mutation with DM in GSD.

Materials and methods

Sample collection

Peripheral blood samples were collected from 47 GSD of both genders aged from 5 months to 14 years, including 10 clinically suspected of having a progressive neurological disease. Dogs were patients from the Veterinary Hospital, University of Turin, and from several private veterinary practitioners. Blood samples were stored at -22°C until analysed.

DNA extraction

Genomic DNA was obtained from blood using the NucleoSpin Blood Quick Pure kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) , according to the manufacturer protocol, and stored at -20°C until use. DNA purity was evaluated by absorbance readings using the NanoDrop-2000 (Thermo Fisher Scientific Inc., Waltham, Massachusetts).

Minisequencing test

The PER reaction is based on the addition of a [F]ddNTP to the 3' end of an unlabeled oligonucleotide (sequencing primer) in the absence of dNTPs in the reaction. Each [F]ddNTP is labeled with a specific fluorescent dye and each sequencing primer is designed immediately adjacent to the diagnosis site.

Preliminary PCR

The PCR primers used in the preliminary PCR tests are shown in Fig.1 (Eurofins MWG Operon, Ebersberg, Germany) [3], PCR reactions were performed in 50 µL volumes consisting of 0.04 U/µL HotStarTaq (Qiagen, Hilden, Germany), 0.2 mM each of dNTPs, 0.5 µM of each primer, and 50-100 ng of DNA template. The PCR profile consisted of an initial activation step at 95°C for 15 min, followed by 35 cycles of 95°C denaturation for 30 sec, annealing at 60°C for 30 sec, and extension

at 72°C for 30 sec. A final extension step of 72°C for 7 min was added to all reactions. Amplifications were carried out using a GeneAmp PCR System 2720 thermal cycler (Applied Biosystems by Life Technologies Italia, Monza, Italy). Amplicons were resolved on a 2.5% agarose gel.

Primer-Extension Reaction (PER)

The PCR products acted as templates for PER after enzymatic clean up. In order to remove primers and unincorporated dNTPs, 2 µL of Exo-Sap (GE Healthcare Europe GmbH, Glattbrugg, Schweiz) were added to 5 µL of PCR products. Tubes were incubated at 37°C for 1 h and at 80°C for 15 min. Based on *SOD1* sequence alignments, the sequencing primer was designed upstream from the *SOD1* mutation site associated with DM (5'- GAACCATTACAGGGCTGACT -3') (Fig. 1) PER reactions were performed following the SNaPshot multiplex Kit protocol (Applied Biosystems by Life Technologies Italia, Monza, Italy) with minor modifications: 2.5 µL of Ready Reaction Mix, 3µL of purified amplicons from the preliminary PCR, diluted to obtain a range of 0.4-0.01 pmol and 0,2µM of sequencing primer in a total volume of 10µL. The reactions underwent 25 single base extension cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C during 30 s. The reactions were carried out in a GeneAmp PCR System 2720 thermal cycler (Applied Biosystems by Life Technologies Italia, Monza, Italy).

Subsequently, a post-extension treatment to remove 5'-phosphoryl group of the [F]ddNTPs helped to prevent unincorporated [F]ddNTPs co-migrating with the extended primers and producing a high background signal. For this purpose, the final volume (10 µL) was treated with 1 unit of Calf Intestine Alkaline Phosphatase (Invitrogen by Life Technologies Italia, Monza, Italy). The tubes were incubated at 37°C for 1 hr and at 65°C for 15 min. Finally, samples were prepared by adding 1 µL of the post-PER product to 12 µL of formamide and 0.2 µL of GeneScan 120 LIZ size standard. Each sample was loaded on the ABI 310 Genetic Analyzer and analyzed using Pop4 polymer, a 47-cm capillary column, the ABI GeneScan E5 Run Module. Parameters for this analysis are the following: injection time 12 s and run time 15 min. Electropherograms were analysed using the GeneScan 3.1.2 software (Applied Biosystems by Life Technologies Italia, Monza, Italy)

Real-Time PCR Allelic Discrimination test

In our study, we develop a Real-Time PCR Allelic Discrimination TaqMan MGB assay based on the *SOD1:c.118G>A* mutation analysis.

Recently, the Real-Time PCR Allelic Discrimination test has been largely used to genotype individual DNA at known mutation site [10].

During amplification, the fluorogenic TaqMan probes anneal specifically to complementary sequences between forward and reverse primer binding sites on the DNA template and they are then degraded owing to the 5'-3' exonuclease activity of DNA polymerase. Once separated from the quencher, the reporter dye emits fluorescence, which is read by the Real-Time PCR system.

1 In the assay here described, the two probes are 100% identical with their respective target
2 sequences; only a single mismatch in the 5' end is observed between them. The contemporary
3 presence of the two probes allows the annealing of both regardless of the allele present because the
4 single mismatch does not impair the probes' annealing to heterologous DNA. During the extension,
5 the 5'-3' Exonuclease activity of the DNA polymerase degrades the probe that is totally identical to
6 the template with a consequent fluorescent emission. On the contrary, the probe that has a mismatch
7 with the sequence is removed without degradation and devoid of fluorescent emission. The post-
8 PCR analysis allows the instantaneous visualization of the results by mean of a specific plot.

9 Primer and probe design

10 The PCR primers [3] were used in conjunction with two MGB probes (Applied Biosystems by Life
11 Technologies Italia, Monza, Italy). One probe was designed to specifically hybridize, with a 100%
12 of identity, to one allele (*SOD1*_ProbeA 5'- 6FAM-CTGACTAAAGGCGAG -3') and the other one
13 to the second allele (*SOD1*_ProbeG 5'- VIC-CTGACTGAAGGCGAGC -3') (Fig. 2).

14 Analysis of diagnostic site

15 Real-time PCR was performed in a 25 µL reaction mixture containing 900 nM of both forward and
16 reverse primers, 200 nM of each probe, 12.5 µL of TaqMan Universal Master Mix (Applied
17 Biosystems by Life Technologies Italia, Monza, Italy), and 30-50 ng of DNA template. Thermal
18 cyclings were performed on a 7300 Real-Time PCR system (Applied Biosystems by Life
19 Technologies Italia, Monza, Italy) under the following conditions: 95°C for 10 min, and 45 cycles
20 of 95°C for 15 s and 60°C for 1 min. All PCR reactions were run in duplicate.

21 Results were obtained by the automatic calling feature of the Allelic Discrimination option in SDS
22 v1.2.1 software. Alleles were assigned to samples by visual inspection of a plot of the fluorescence
23 (Rn) from the A probe versus Rn from G probe generated from the post-PCR fluorescence read.

1 *Sequencing*

1
2 2 DNA sequencing was carried out as a confirmatory test. Amplified fragments of the preliminary
3
4 3 PCR of a homozygous AA dog and a homozygous GG animal were cloned into the pDRIVE
5
6
7 4 Cloning Vector using QIAGEN PCR Cloning Kit (GE Healthcare Europe GmbH, Glattbrugg,
8
9
10 5 Schweiz). Clones were subsequently submitted to RFLP analysis using Hae III to choose a clone
11
12 6 that contained the diagnostic site. These cloned fragments were cycle sequenced on an ABI PRISM
13
14 7 310 Genetic Analyser (Applied Biosystems by Life Technologies Italia, Monza, Italy) , using the
15
16
17 8 ABI Prism BigDye terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems by Life
18
19 9 Technologies Italia, Monza, Italy), version 1.1 by the dideoxy chain termination method with
20
21
22 10 fluorescence dye terminators. The sequencing on both strands was performed using the two M13
23
24 11 vector primers. The resulting sequences were compared and aligned with 'ClustalW' program[11].
25
26

27 12
28

29 **Results**

31 *Minisequencing test*

32 14
33
34 15 The primers used for the preliminary PCR successfully amplified a 79 bp fragment. For
35
36 16 homozygote dogs the PER analysis gave rise to a peak of the color that is specific to the nucleotide
37
38
39 17 present on template, green for A and blue for G. In heterozygote animals both peaks, green and
40
41 18 blue, were generated (Fig. 3). On 47 GSDs analysed by minisequencing, two homozygote
42
43
44 19 individuals for the mutant allele (AA) were detected, 12 heterozygous GA, all other dogs were
45
46 20 homozygote for the wild allele (GG).
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Real-Time PCR Allelic Discrimination test

Real-Time PCR Allelic Discrimination test confirmed the genotypes obtained with PER. A representative plot used to genotype individuals is displayed in Fig 4. The Real Time test was applied to all 47 individuals. Two dogs were confirmed to be A/A, 33 dogs were G/G and 12 G/A. In the examined cohort the allele frequencies were 0.17 and 0.83 for A and G, respectively.

Sequencing

Ultimately, the sequencing of the amplicons obtained from the preliminary PCR confirmed the reliability of both techniques for genotyping the *SOD1:c118G>A* polymorphism in dog.

Association between DM and SOD1c.118G>A

Out of the 47 dogs examined in this study, 10 were clinically suspected to have DM. Therefore, depending on availability of tissues and clinical information, diagnoses of DM were made on the basis of criteria of varying stringency. Only in one case the clinical diagnosis was confirmed by postmortem investigations (histopathology of the spinal cord tissue) (data not shown). It was a male, 8 years old, and it resulted to be an A/A homozygote. In another clinically suspected dog, a male 14 years old, heterozygote A/G, the histopathological examination of the medulla showed only moderate axon and myelin degenerative changes limited to the thoracic segments. One more A/A clinically suspected animal is still alive. For all the other clinically suspected dogs (1 A/G and 6 G/G) the diagnosis was not confirmed by histopathological examination of the medulla or unfortunately the necropsy was not carried out.

From the other 37 dogs not suspected to have DM, none was homozygote A/A, 27 were homozygotes G/G, and the other heterozygotes.

Discussion

In a previous study *SOD1* in normal and DM affected dogs was sequenced and a G to A transition in exon 2, predicting an E40K missense mutation, was observed [3]. Homozygosity for the A allele

was highly associated with DM in five dog breeds (GSD, Pembroke Welsh Corgi, Boxer, Rhodesian ridgeback, and Chesapeake Bay retriever); therefore the A allele was recognized as a major genetic risk factor for the disease. Recently, two genotyping assays for the *SOD1* mutation, using conventional and Real Time PCR methods have been proposed [12]. The aim of our study was to develop a test for rapid screening and identification of *SOD1:c.118G>A* mutation carriers in dogs using two different approaches: a minisequencing test and a Real-Time PCR Allelic Discrimination test. Both approaches are effective and efficient, all genotypes were clearly determined without non-specific allelic amplification. Costs for consumables and reliability of results are similar. Real Time Allelic Discrimination test provides several advantages because it does not require any post-amplification step and utilizes 96-well format plates that can be read in about 5 min, enabling a rapid diagnosis of large numbers of samples. On the other hand, the reads of the minisequencing test have a precision comparable to that of the sequencing. This technique can be suitable for automation and high-throughput testing for laboratories equipped with an ABI sequencer. In comparison with sequencing, the minisequencing has the advantage of being quicker, allowing an immediate interpretation of the results. Moreover, it may be implemented for the multiplex analysis of several mutation sites. Recently, a novel *SOD1* missense mutation, associated with DM, has been described in the Bernese Mountain Dog breed [13]. It would be interesting to implement a minisequencing test for the concurrent genotyping of multiple SNPs. Ultimately, the choice of the more suitable approach depends on laboratory disposable equipment.

Representatives of 217 different dog breeds were genotyped at *SOD1:c.118G>A* and 115 of them were found to segregate the A allele [14]. The Wire Fox Terrier had the highest *SOD1:c.118A* allele frequency (0.98). Likewise, in a survey on Pembroke Welsh Corgi, the mutant allele was found to be prevalent (0.68) [12]. In the present investigation, GSD shows a frequency of the mutant allele equal to 0.17, quite high being a high-risk allele. Because canine DM has a late onset in adulthood and homozygous mutant dogs are likely as fertile as other genotypes, the natural selection is mild

1 and the mutant allele may reach high frequencies. A diagnostic test, easy to implement, may
2 contribute to control the gene diffusion in populations.

3
4 According to the hypothesis of Awano [3], the DM seems to be an age-related, incompletely
5 penetrant disease. As far as the gene action is concerned, a model of inheritance based on dominant
6 allele, as in humans, may not be excluded. In fact, not only the homozygote dogs exhibit clinical
7 signs, but also heterozygote animals develop the pathology, although very slowly. Therefore, the
8 clinical signs become apparent solely in dogs surviving longer the usual canine life span.

9 **Acknowledgements**

10 The authors thank Alessandra Sereno for technical support.

11 **Declaration of conflicting interests**

12 The authors declared no potential conflicts of interest with respect to the research, authorship,
13 and/or publication of this article.

References

1. Coates JR, Wininger FA (2010) Canine degenerative myelopathy. Vet Clin North Am Small Anim Pract 40:929-50.
2. March PA, Coates JR, Abyad R, et al.(2009) Degenerative myelopathy in 18 Pembroke Welsh Corgi dogs. Vet Pathol 46:241-250.
3. Awano T, Johnson GS, Wade C, et al.(2009) Genome-wide association analysis reveals a *SOD1* missense mutation canine degenerative myelopathy that resembles amyotrophic lateral sclerosis. Proc Natl Acad Sci 106:2794-2799.
4. Jonsson PA, Ernhill K, Andersen PM, et al.(2004) Minute quantities of misfolded mutant superoxide dismutase-1 cause amyotrophic lateral sclerosis. Brain 127:73-88.
5. Shaw BF, Durazo A, Nersissian AM, et al. (2006) Local unfolding in a destabilized, pathogenic variant of superoxide dismutase 1 observed with H/D exchange and mass spectrometry. J Biol Chem 281:18167-76.
6. Rakhit R, Chakrabartty A.(2006) Structure, folding, and misfolding of Cu,Zn superoxide dismutase in amyotrophic lateral sclerosis. Biochim Biophys Acta 1762:1025-37.
7. Marucci G, Morandi L, Bartolomei I, et al.(2007) Amyotrophic lateral sclerosis with mutation of the Cu/Zn superoxide dismutase gene (*SOD1*) in a patient with Down syndrome. Neuromuscul Disord 17:673–676.
8. Rosen DR, Siddique T, Patterson D. et al.(1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 362:59–62.
9. Pasinelli P, Brown RH.: 2006, Molecular biology of amyotrophic lateral sclerosis: Insights from genetics. Nat Rev Neurosci 7:710–723.
10. Stopińska K, Grzybowski T, Malyarchuk BA, et al.(2006) Optimization of the Y831C mutation detection in human DNA polymerase gamma by allelic discrimination assay. Acta Biochim Pol 53:591-5.

11. Thompson JD, Higgins DG, Gibson TJ(1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673-4680.
12. Chang HS, Kamishina H, Mizukami K, et al.(2013) Genotyping Assays for the Canine Degenerative Myelopathy-Associated c.118G>A (p.E40K) Mutation of the *SOD1* Gene Using Conventional and Real-Time PCR Methods: A High Prevalence in the Pembroke Welsh Corgi Breed in Japan. J Vet Med Sci 2013 Jan 18. (Epub ahead of print)
13. Wininger FA, Zeng R, Johnson GS et al.(2011) Degenerative myelopathy in a Bernese Mountain Dog with a novel *SOD1* missense mutation. J Vet Intern Med 25: 1166-1170
14. Zeng R, Coates JR, Hansen L, et al.(2012) The distributions of two *SOD1* missense mutations in the pet dog population and their association with canine degenerative myelopathy, a model for amyotrophic lateral sclerosis. American Society of Human Genetics (ASHG)'s 62nd Annual Meeting, 6-10, November 2012 San Francisco, CA.

Legends for figures

Fig. 1 Primers design for minisequencing (preliminary PCR primers in bold and PER primer in grey). The bases in the box correspond to the *SOD1* mutation site.

Fig. 2 Primers and probes design for Real-Time PCR Allelic Discrimination (primers in bold and probes in grey). The bases in the box correspond to the *SOD1* mutation site.

Fig. 3 Minisequencing patterns for the three genotypes: G/G (a), A/A (b), and G/A (c).

Fig. 4 Real-Time PCR Allelic Discrimination plot. Fluorescence of G (x-axis) and A (y-axis) probes: no-template controls (squares), G/G (circles), A/A (diamonds), and G/A (triangles).

Figure 1
[Click here to download high resolution image](#)

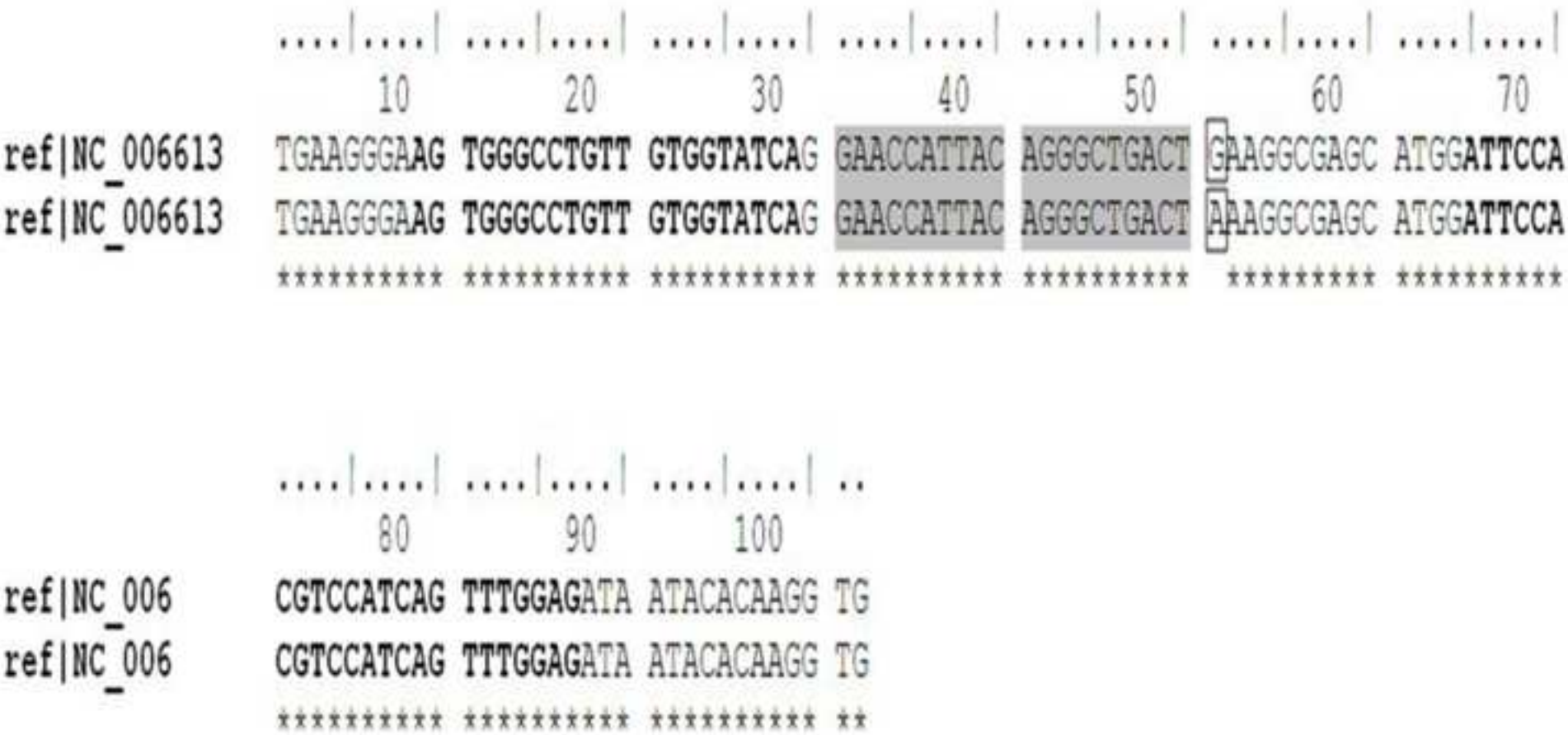


Figure 2
[Click here to download high resolution image](#)

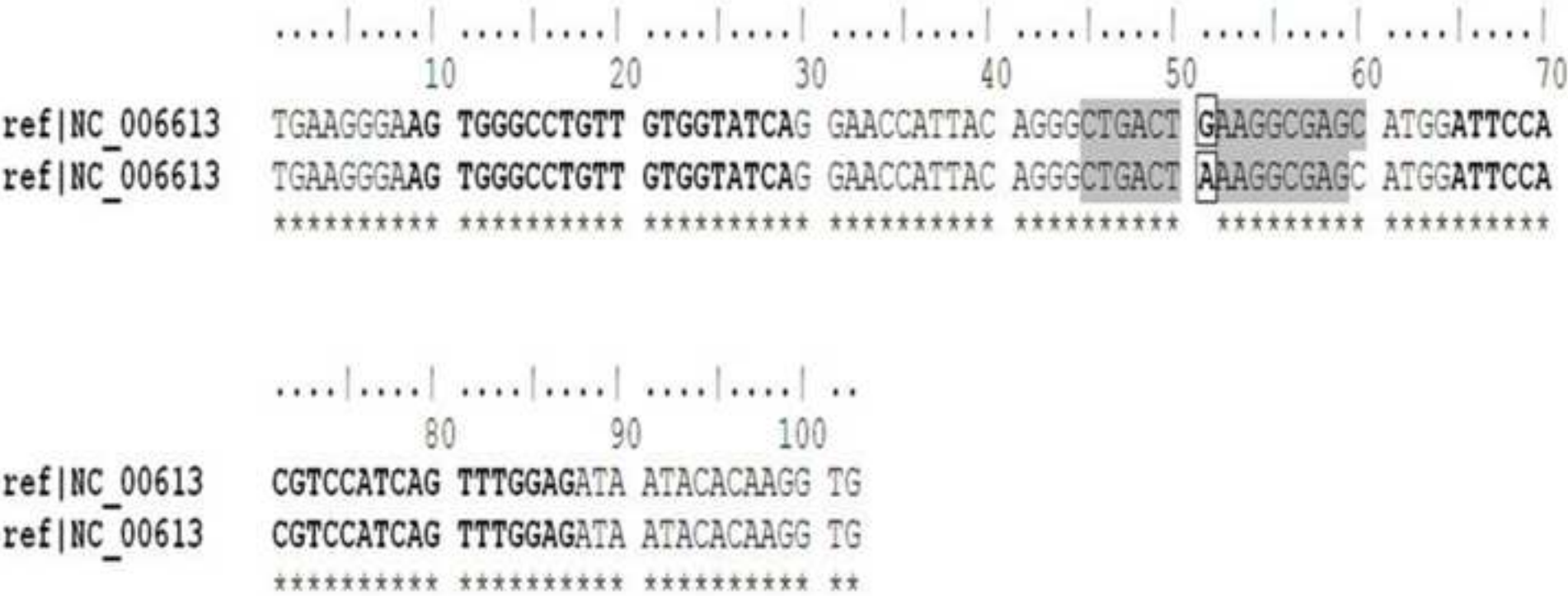


Figure 3
[Click here to download high resolution image](#)

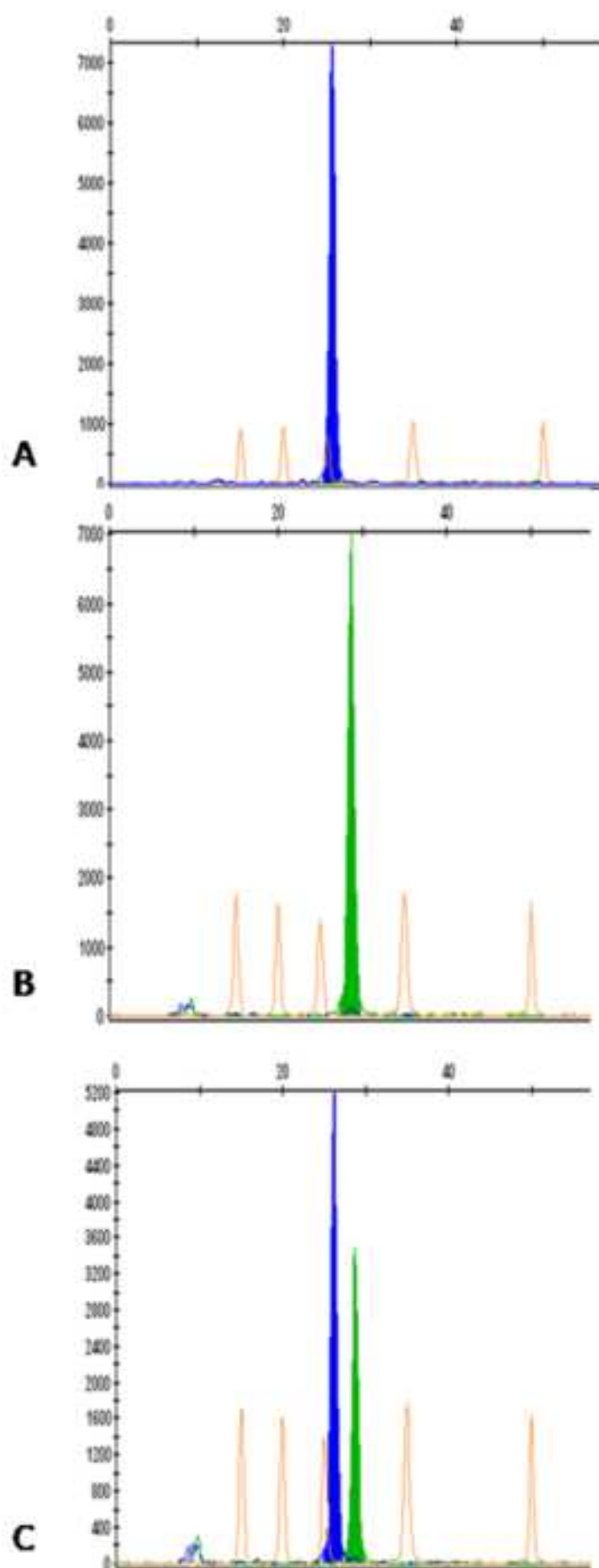


Figure 4
[Click here to download high resolution image](#)

